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EFFECT OF PROTEIN PHOSPHORYLATION ON THE FUNCTIONAL ACTIVITY OF PHOTOSYSTEM 2 UNDER PHOTOINHIBITORY CONDITIONS

Katia Georgieva

Acad. M. Popov Institute of Plant Physiology, Acad. G. Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria

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Summary. The effect of protein phosphorylation on the functional activity of PS2 under control and photoinhibitory conditions has been investigated. The time course of phosphorylated and non-phosphorylated PS2 enriched membranes (BBYs) has been monitored by chlorophyll fluorescence using a PAM fluorimeter. The results show that photochemical activity did not change during treatment in the dark but decreased sharply as a result of high light (2000 μmol.m^{-2}.s^{-1}) in both phosphorylated and non-phosphorylated preparations. No difference could be detected between the polypeptide profiles of control and phosphorylated BBYs as judged by SDS-PAGE and Western blotting. The results suggest that the decrease of photochemical activity took place prior to protein degradation. In order to check if protein phosphorylation had some protective effect at lower light intensities we subjected the BBYs to 1000 μmol.m^{-2}.s^{-1}. The experiment showed that the photochemical activity of PS2 was slightly more resistant to photoinhibiiton in the phosphorylated compared with the non-phosphorylated samples. Again, there was no difference between the polypeptide profiles of the phosphorylated and non-phosphorylated preparations even after one hour of exposure to illumination. Overall the results indicate that the phosphorylation of PS2 proteins does not significantly influence the rate of photochemical inactivation of PS2 but rather may serve to regulate the conformational state of the complex.

Key words: photoinhibition, protein phosphorylation, photosystem 2, chlorophyll fluorescence

Abbreviations: PS2 – photosystem 2; BBYs – PS2 enriched membranes; SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis;

Q_A and Q_B – primary and secondary electron acceptors of PS 2; F_m – maximal chlorophyll fluorescene; F_0 – ground chlorophyll fluorescence; F_v – variable chlorophyll fluorescence.

Introduction

Exposure of plants to strong light induces inhibition of photosynthesis. It is generally accepted that photosystem 2 (PS2) is predominantly affected by photoinhibition (Powles, 1984). PS2 functions as a light-dependent water-plastoquinone-oxydoreductase and consists of at least 25 proteins. D1 and D2 reaction centre proteins which bind P680 and other redox components of PS2, the water-oxidising complex and the light harvesting chlorophyll proteins have a central role in the function of PS2.

Light harvesting chlorophyll *a*/*b* proteins of PS2 (LHC2, 25 and 27 kDa) and four PS2 core proteins (D1, D2, 43 kDa chlorophyll-binding protein and 9 kDa psb H gene product) have been shown to be reversibly phosphorylated (Bennet, 1991; Allen, 1992). The phosphorylation/dephosphorylation cycle of LHC2 is well documented and it has been postulated to function in state 1 – state 2 transitions. Total LHC2 is found preferentially (75–80%) in stacked membranes, where it functions as a peripheral antenna for PS2. However, a part of LHC2 is now recognised to be mobile and to migrate laterally from the stacked to the unstacked regions. The mobile fraction is enriched in phosphorylated LHC 2. The available data suggest that the phosphorylation of 25 and 27 kDa LHC 2 units in stacked membranes leads to their detachment from PS2 and consequently to a decrease in energy transfer to PS2 and some of these detached LHC2 units (mainly 25 kDa) migrate from the stacked to the unstacked membranes, where they can act as antennae for PS1. It has been suggested that a major effect of LHC2 phosphorylation is on PS2 heterogeneity, either to convert α-centres into β-centres or to cause complementary changes in the absorption cross-section of PS2α and PS2β (Berens et al., 1985).

The significance of PS2 core phosphoproteins is not yet clear. Phosphorylation of the D1 and psb H gene product has been proposed to be involved in photoinhibition of photosynthesis (Aro et al., 1992; Giardi, 1993).

The aim of our investigation was to analyse the influence of PS 2 protein phosphorylation on its sensitivity to photoinhibition.

Material and Methods

Thylakoids were isolated from 12-day-old pea plants (*Pisum sativum*, cv. Ran) after incubating them in dark for 12 h to promote maximum dephosphorylation. They were phosphorylated following a modified version of the procedure of Pramanic et al.

(1991). The isolated thylakoids were suspended in a medium containing 50 mM HEPES/KOH, pH 8.0, 10 mM $MgCl_2$ and 100 mM sucrose to a concentration of 400 µg chlorophyll/ml. Thylakoids were phosphorylated at room temperature by illumination for 25 min with 300 $\mu mol.m^{-2}.s^{-1}$ in the presence of 0.4 mM ATP. To inhibit phosphatase activities NaF was added at a concentration of 20 mM. Phosphorylation was stopped by centrifugation at 0°C followed by ressuspension of the thylakoids into a medium containing 50 mM MES, pH 6.0, 5 mM $MgCl_2$, 15 mM NaCl, 0.5 g/l Na ascorbate and 10 mM NaF. PS 2 enriched membranes (BBYs) were obtained by treating thylakoids with 2% Triton X-100 for 15 min on ice in the dark followed by differential centrifugation according to Berthold, Babcock and Yocum (1981).

High light treatment were performed in a stirred glass cuvette at 25°C with a chlorophyll concentration of 100 µg/ml in 25 mM MES, pH 6.5, 0.3 M sucrose, 10 mM NaCl, 5 mM $CaCl_2$, 10 mM $NaHCO_3$ and 10 mM NaF.

Gradient gels (10–17% polyacrylamide) containing 6 M urea were used for analysis of the polypeptide composition of samples as described by Kruse et al. (1995). Gels were either silver stained for protein visualisation or prepared for western immunobloting. Profiles of separated proteins were transferred onto nitrocellulose and detecting using specific antibodies against the main PS2 proteins (available in Biochemistry Department, Imperial College, London).

The chlorophyll fluorescence parameters were measured using a pulse modulation fluorometer (PAM 101, H. Walz, Germany). Experimental data were processed statistically after Student.

Results

The results obtained showed that the control BBYs had a very good photochemical activity. The value of the F_v/F_m ratio, which is a measure of PS2 effectiveness in the primary photochemical reactions was about 0.80–0.81, i.e. almost the same as in leaves and thylakoids (0.82–0.83). The initial PS2 activity of phosphorylated BBYs was a little bit lowed than those of control. Photochemical activity of PS2 did not change during treatment in the dark but it decreased sharply as a result of high light treatment in both phosphorylated and non-phosphorylated preparations (data not shown).

Fig. 1 shows the influence of high light treatment at 2000 $\mu mol.m^{-2}.s^{-1}$ on different chlorophyll fluorescence parameters measured in phosphorylated and non-phosphorylated BBYs. Cold ATP was included to reduce dephosphorylation during photoinhibitory illumination of phosphorylated preparations.

Ground fluorescence F_0, which is a measure for the initial distribution of the excitation energy to PS2 and the effectiveness of excitation capture in P680, increased as a result of high light stress by 30% after 15 min of treatment. All other parameters,

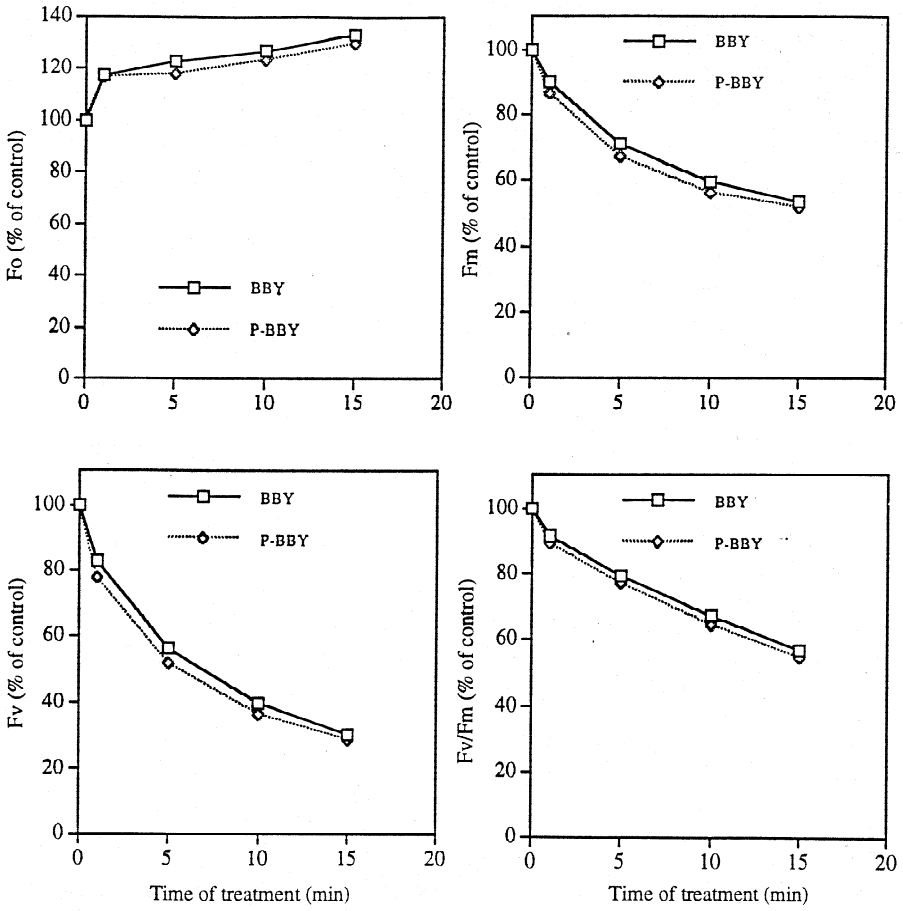

Fig. 1. Influence of high light treatment at 2000 µmol.m^{-2}.s^{-1} on the chlorophyll fluorescence parameters measured in phosphorylated (P-BBYs) and non-phosphorylated BBYs at 25°C

characterising the functional PS 2 activity, decreased: maximal fluorescence F_m decreased by 48%, variable fluorescence F_v by 70% and the ratio F_v/F_m by 45%. The decline of variable fluorescence resulted from both an increase of F_0 and decrease of F_m. The value of all fluorescence parameters decreased a little bit higher in phosphorylated BBYs but the differences were not so big.

It has been found a good correlation between the increase in F_0 and the loss of the D1 protein (Rintamaki et al., 1994). Our results showed that F_0 increased by 30 and 33% in phosphorylated and non-phosphorylated BBYs respectively after 15 min of treatment at 2000 µmol.m^{-2}.s^{-1} but there was not any difference in the polypeptide profiles of control and phosphorylated BBYs on SDS-PAGE. Blotting of the proteins on the nitrocelulose membrane and using the antibodies against the main PS 2 prote-

ins did not show significant differences in protein stoichiometry of phosphorylated and non-phosphorylated preparations even after 30 min of treatment (data not shown). It should be mentioned that the photochemical efficiency of PS 2 was almost fully inhibited at that conditions. Those results suggested that the decreasing of the photochemical activity of PS 2 took place before protein degradation at high light conditions. Significant differences in the polypeptide profile were induced after 60 min of treatment at 2000 μmol.m^{-2}.s^{-1} (Fig.2). The results obtained showed that the amount of D1 and CP 43 were significantly reduced**.** Moreover, phosphorylated CP 43 seemed to be more stable under high light conditions compared to its non-phosphorylated form (Fig. 3). It should be mentioned that an aggregation band containing CP 43 occurred during that conditions whereas the aggregated D1/D2 heterodimer almost disappeared with time of illumination. The content of D2 protein, CP 47, CP 26 and α-subunits of cyt b_{559} showed considerably smaller reductions. The content of the other Lhcb proteins remained unchanged. Summarising the results obtained it can be said that 60 min high light treatment resulted in partial losses of the main PS 2 proteins.

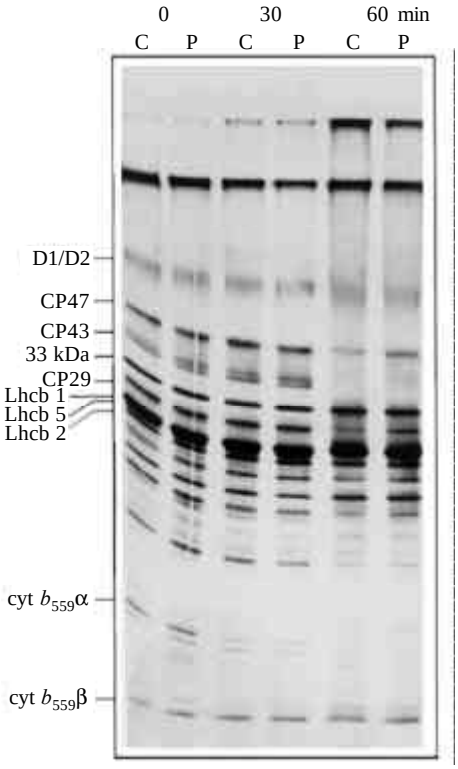

Fig. 2. Silver stained SDS-PAGE of control (C) and phosphorylated (P) BBYs after 0, 30 and 60 min of high light treatment at 2000 μmol.m^{-2}.s^{-1} and 25°C

Illumination of BBYs generates a protein of 41 kDa molecular mass. Using immunobloting it was confirmed that the protein was an cross-linking product of the D1 protein and α-subunits of cyt b_{559} (data not shown).

As it was already mentioned the photochemical activity of PS2 in phosphorylated and non-phosphorylated BBYs was approximately the same after treatment at 2000 μmol.m^{-2}.s^{-1}. In order to see if protein phosphorylation had some protective effect at lower light intensity we treated the BBYs at 1000 μmol.m^{-2}.s^{-1}. The result showed that F_v/F_m ratio as well as F_m and F_v of the phosphorylated BBYs were about 5% higher than those in control BBYs at those conditions (Fig. 4). There was not only difference between the polypeptide profiles of both phosphorylated and non-phosphorylated preparations even after 1 h of treatment.

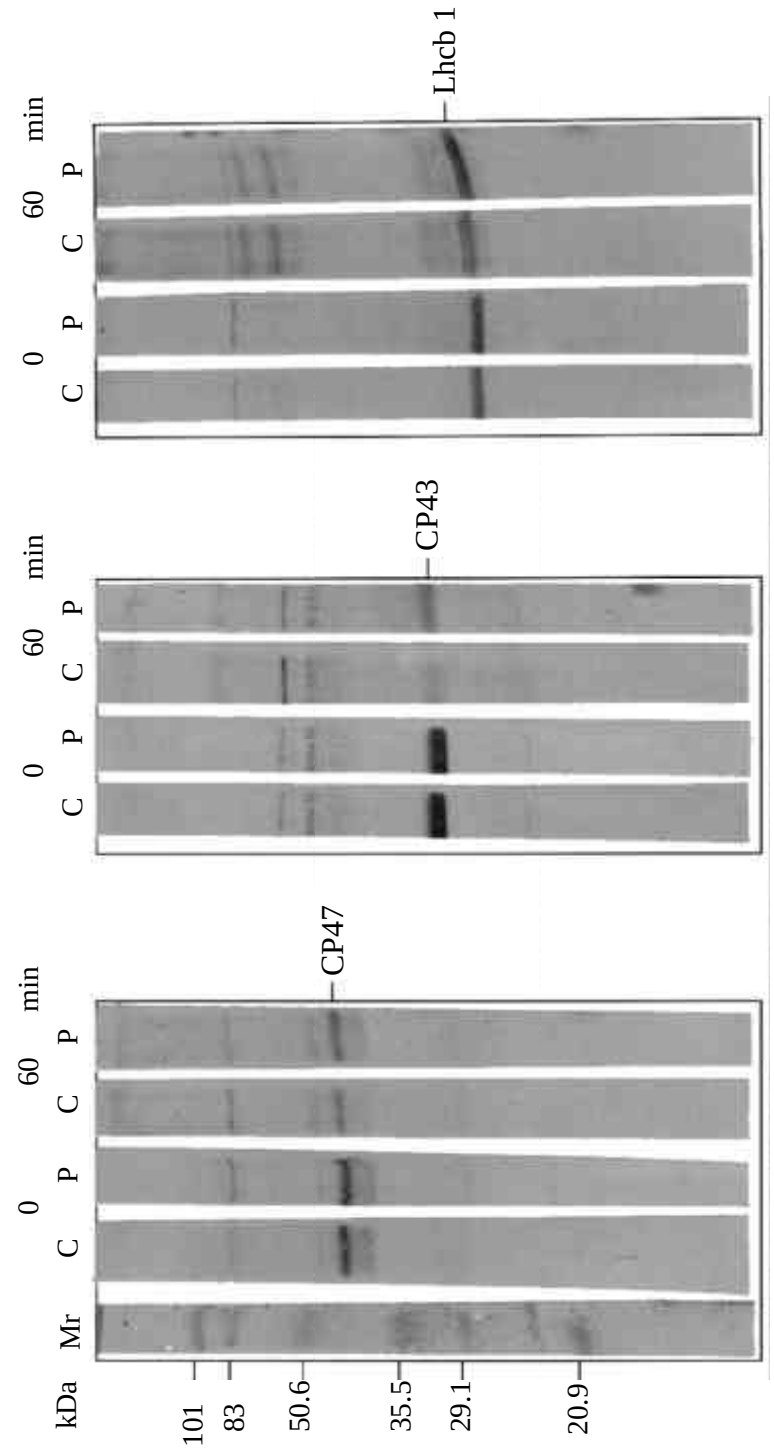

Fig. 3. Immunoblot of CP47, CP43 and Lhcb1 in control (C) and phosphorylated (P) BBYs after 0 and 60 min of high light treatment at 2000 µmol.m^{-2}.s^{-1} and 25°C

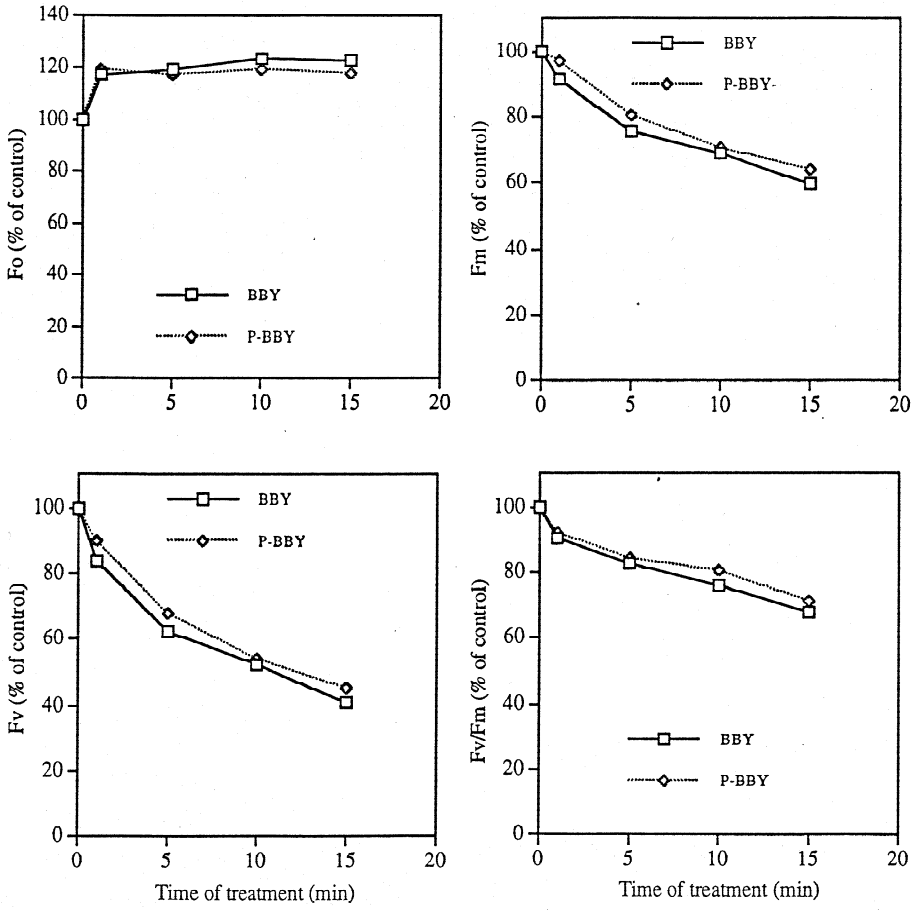

Fig. 4. Influence of high light treatment at 1000 µmol.m^{-2}.s^{-1} on the chlorophyll fluorescence parameters measured in phosphorylated (P-BBYs) and non-phosphorylated BBYs at 25°C

Discussion

In this work we investigated the effect of high light treatment on PS 2 activity in phosphorylated and non-phosphorylated BBYs. We demonstrated that the damaging of electron transport reactions through PS 2 took place before protein degradation. Several experimental studies have shown that the photoinactivation can be initiated both from the acceptor and donor side of PS 2. As proposed by Barber and Andersson (1992) the acceptor side inhibition occurs under high light conditions which can fully reduce the plastoquinone pool. Because of the lack of oxidised quinone, the Q_B binding site thus left empty. Under this conditions the high light intensity favours double reduction of Q_A, which is than protonated and the Q_AH_2 subsequently vacates the Q_A

binding site. With the Q_A site empty, the radical pair $P680^+Pheo^-$ can be formed and will recombine generating the triplet state of P680. This, it was proposed, reacts with oxygen to form singlet oxygen. The toxic effect of $^1O_2$ is then thought to destroy the chlorophyll P680 which subsequently leads to D1 degradation.

It was proposed that the donor side mechanism would occur under conditions where electron donation from water could not keep with electron withdrawal on the acceptor side. This will lead to an increase in the lifetime of $P680^+$ which has a redox potential of more than 1 V, would be able to extract electrons from its surrounding environment causing damage to both the chromophores and protein.

Rintamaki et al. (1994) have found a good correlation between the increase in F_0 and the loss of the D1 protein. Our results showed that F_0 increased by 30–33% after 15 min of treatment at 2000 $\mu mol.m^{-2}.s^{-1}$ but there was not any changes in D1 at that conditions (Fig. 1). F_0 sharply increase in light stress conditions causing irreversible damage of the photosynthetic apparatus. The slightly lower trapping efficiency of dissipative centres would explain the rise in the ground fluorescence, often observed under photoinhibitory conditions (Critchley and Russel, 1994). According to Aro et al. (1993) delaying of D1 degradation would result in the accumulation of inactive PS 2 centres containing damaged D1 protein at high light conditions. The damaged PS 2 centres might still function as an energy trap, and if the trapped energy is converted to heat, the F_0 state of damaged PS 2 does not increase (Giersch and Krause, 1991).

Our results showed that prolonged high light treatment (60 min at 2000 $\mu mol.m^{-2}.s^{-1}$) induced a partial losses of PS 2 core proteins. We found a reduction in the amount of D1 and CP 43 and in D2, CP 47, CP 26 and α-subunits of cyt b_{559} to a lesser extent.

Photoinhibition of PS 2 electron transfer induces damage to the D1 protein and subsequent recovery of PS 2 function requires degradation and resynthesis of the D1 protein (Ohad et al., 1985; Aro et al., 1993, 1994; Rintamaki et al., 1994). Thus the rapid degradation of the D1 protein under high light conditions is one of the first steps in the restoration of the photochemical efficiency of PS 2 (Rintamaki et al., 1995). Schuster et al. (1988) showed that D1 was the main PS 2 polypeptide, which was degradating during photoinhibition. At excess irradiance the stability of the D2 protein was also impaired but to a lesser extent than that of D1 protein. While the other PS 2 polypeptides – CP 43, CP 47, CP 29 and the polypeptides of the water-oxidising complex were fairly stable. Schmid et al. (1995) have found a net loss of D1 protein under high light treatment which was accompanied by comparable losses of other proteins of the PS 2 core – D2, CP 43, CP 47. It was suggested that the CP 43 subunit is possibly involved in D1 protein degradation (Salter at al., 1992). The minor cab proteins CP 26 and CP 29 showed considerably smaller reductions. LHC 2 proteins were not reduced correspondingly, indicating that these complexes are less affected by prolonged high light.

We found 41 kDa cross-linking product between D1 and α-subunits of cyt b_{559} after 60 min treatment at 2000 $\mu mol.m^{-2}.s^{-1}$. Barbato et al. (1995) suggest that it may be an intermediate before the light induced cleavage of the D1 protein.

It was suggested that phosphorylation of PS 2 proteins protects against acceptor-side photoinhibition by suppressing free radical formation. However, our results showed that the photochemical activity of PS 2 in phosphorylated and non-phosphorylated BBYs was approximately the same after treatment at 2000 μmol.m^{-2}.s^{-1}. Measurements at lower light intensity (1000 μmol.m^{-2}.s^{-1}) showed a little bit higher photochemical activity in phosphorylated preparations but the difference was not so big (about 5%). Our data are in agreement with those of Koivuniemi et al. (1995) who found that the PS 2 activity of both phosphorylated and unphosphorylated spinach thylakoids was equally susceptible to high-light-induced (2000 μmol.m^{-2}.s^{-1}) acceptor-side photoinactivation.

In vitro experiments have indicated that the phosphorylation form of D1 protein is degradated under photoinhibitory illumination more slowly than non-phosphorylated D1 protein (Aro et al., 1992; Kettunen et al., 1992). It was found that the phosphorylated form of the D1 protein is quite a poor substrate for degradation in isolated thylakoid membranes and PS 2 core particles. It was proposed (Rintamaki et al., 1995; Critchley and Russel, 1995) that phosphorylation of D1 protein may also change the conformation of the Q_B niche in such a way that the degradation of D1 is reduced. Koivuniami et al. (1995) suggest that phosphorylated and damaged D1 protein is an intermediate stage in turnover that avoids excess D1 protein degradation in the absence of D1 synthesis. They favour a mechanism where protein phosphorylation renders D1 protein unavailable to proteolitic attack because phosphorylated D1 and D2 proteins have an altered conformation or otherwise unavailable recognition site.

Summarising the results obtained it can be concluded that phosphorylation of PS 2 proteins did not influence significantly photochemical activity of this photosystem. Our data rather support the suggestion that phosphorylation of PS 2 proteins plays a role in regulating their structure and to keep them from further degradation.

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